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The transformation of protoplasts of *Leptosphaeria maculans* to hygromycin B resistance

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Summary. Conditions are described for the efficient isolation and regeneration of protoplasts of a fungal pathogen of brassicas, *Leptosphaeria maculans*. Treatment of the protoplasts with DNA of the plasmid pAN7-1 (containing an *E. coli* hygromycin phosphotransferase gene with *Aspergillus nidulans* expression signals) and plating under selective conditions resulted in the formation of hygromycin B-resistant colonies. Southern blot analysis of resistant colonies indicated that single copies of the plasmid had integrated into different sites in the genome. In twelve of the transformants analysed so far, the integration is stable through mitosis. The demonstration of efficient transformation is an essential first step in the molecular analysis of pathogenicity of this commercially important pathogen.

Key words: Brassica – Oil-seed rape – *Phoma lingam* – Stem canker – Ascomycetes

Introduction

Leptosphaeria maculans (syn. *Phoma lingam*) causes the disease blackleg of brassicas (Gabrielson 1983). The interaction is relatively complex and resistance may be expressed at a number of stages of infection. Leaf infection leads to the development, in susceptible cultivars, of a leaf spot. Subsequently hyphae may penetrate the petiole and colonise the stem causing a canker (Hammond et al. 1985). The leaf infection is, at least initially, biotrophic whereas the stem disease is wholly necrotrophic. This complex pattern indicates that the coordinated expression of a large number of genes is likely to be involved in the pathogenesis. The ultimate aim of this study is to isolate and characterise these genes. This requires the development of molecular genetic methods for the pathogen, namely the isolation and regeneration of protoplasts and the establishment of an efficient transformation procedure.

Protoplasts of filamentous fungi were widely used in physiological and genetic techniques (Peberdy 1979, 1987). Their use in transformation is of particular importance and very rapid developments are occurring (Mishra 1985). The transformation of plant pathogenic species is at an early stage. As these species have often been minimally characterised genetically the simplest approach to the selection of transformants is to use a plasmid containing a positively-selectable gene. Turgeon et al. (1985) used the *Aspergillus nidulans amdS* gene conferring acetamide utilisation when transforming *Cochliobolus heterostrophus*. The development of vectors, conferring antibiotic resistance may prove to be of more general value. Hygromycin B is an aminoglycoside antibiotic which interferes with both 70s and 80s ribosomes (Pittinger et al. 1953). Vectors based on an *E. coli* hygromycin phosphotransferase (hph) gene have been used to transform several filamentous fungi including *Cephalosporium acremonium* (Queener et al. 1985), *Cochliobolus heterostrophus* (Yoder et al. 1985), *Aspergillus nidulans* and *A. niger* (Punt et al. 1987) and *Fulvia fulva* (Oliver et al. 1987). Here we report the conditions for the efficient transformation of *Leptosphaeria maculans* protoplasts to hygromycin B resistance.

Materials and methods

Strains and media

L. maculans (Desm.) Ces. and de Not, isolate Lm11 (Mithen et al. 1986), accession No. 558A, was obtained from D. F. Hum-

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pherson-Jones at the National Vegetable Research Station, Wellesbourne, Warks, England, and maintained as described.

Mycelium was recovered from sterile oat kernels (McGee and Petrie 1978) and maintained on V8-juice agar. Pycnidiospores were routinely obtained by inoculation onto V8 plates, harvested after 10 days and were stored in 15% glycerol at −80 °C.

Preparation of protoplasts

Approximately 5 x 10^8 spores were thawed at room temp. and inoculated into 200 ml potato dextrose broth in a 1 l flask. This was incubated at 28 °C for 18 h on a rotary shaker at 200 rpm. Germlings and sparce mycelial growth were harvested by centrifugation at 3,000 g for 5 min. 100 mg of the damp pellet was resuspended by repeated pipetting in 10 ml of enzyme solution containing 0.8 M NaCl, 7.5 mg Novozym 234 (batch No. 1906) (Novo Industri, Copenhagen), 20% w/v β-glucuronidase, 20 mg/ml driselase and 50 μg/ml chitinase (all from Sigma) in a 50 ml flask. The suspension was incubated at 28 °C with shaking (100 rpm) for 1–2 h, on a reciprocal shaker.

The entire contents of the incubation was pelleted by centrifugation, 3,000 g for 5 min. This pellet was resuspended in 0.8 M NaCl in a 15 ml Corex tube by repeated, but careful pipetting. The suspension was underlayed with 2 ml of 1.0 M $MgSO_4$. After centrifugation at 3,000 g for 5 min in a Sorvall HB-4 swing out rotor, the protoplasts were collected from the interface in a volume of 1 ml. 4 ml of 0.8 M NaCl was added to the suspension, and the protoplasts were pelleted by centrifugation at 5,000 g for 10 min. The pellet was resuspended in 0.5 ml NTC buffer (0.8 M NaCl, 10 mM Tris/HCl pH 7.5, 10 mM $CaCl_2$).

Transformation protocol

The transformation protocol was modified from those of Yelton et al. (1984) and Vollmer and Yanofsky (1986). Plasmid pAN7-1 (Punt et al. 1987) (50 μg in 13 μl) was mixed with 2 μl spermidine (50 mM in MTC; 1 M $MgSO_4$, 10 mM Tris/HCl pH 7.5, 10 mM $CaCl_2$) and 5 μl heparin (5 mg/ml) in MTC. To this was added ~ 2 x 10^6 protoplasts in 0.1 ml NTC. These were mixed by inversion and left on ice for 30 min. Polyethylene glycol 4000 (20% w/v in 10 mM Tris/HCl pH 7.5 and 10 mM $CaCl_2$) was added in three aliquots of 200, 200, and 800 μl with gentle rolling after each addition. Following incubation at room temperature for 20 min, 3 ml of molten potato dextrose agar (47 °C) containing 1.2 M sucrose was added to 1 ml of each transformation mix and overlayed onto plates containing 15 ml of potato dextrose agar supplemented with sucrose. These plates were incubated for 3–4 h before overlaying with hygromycin B (250 μg/ml) in 5 ml of potato dextrose agar containing 1.2 M sucrose as an osmotic support.

The remainder of the transformation mix was used to make dilutions of 10^{-1} down to 10^{-4} in 0.8 M NaCl. These were included in overlays on non-selective plates to check protoplast viability. Spore contamination was checked by plating on unstabilised plates.

DNA manipulations

DNA was prepared from transformants essentially as described by Raeder and Broda (1985) except that fresh mycelium was used as starting material.

Restriction enzyme digestions and transfer of DNA to nitrocellulose filters were performed according to standard protocols (Maniatis et al. 1983). Photobiotinylated pAN7-1 was hybridised to the filters and detected using the manufacturers instructions (Clontech, CA, USA).

Results

Preliminary experiments had indicated that the minimum inhibitory concentration of hygromycin B for protoplasts of *Leptosphaeria maculans* was about 10 μg/ml.

Colonies appeared on hygromycin B plates after 4–5 days. The frequency was about 60 colonies/μg DNA, or 3 colonies/2 x 10^3 protoplasts. Protoplast regeneration frequencies on non-selective plates were between 5 and 10% and after treatment with DNA in the presence of P.E.G. were between 2 and 5%. Protoplasts that were not treated with DNA failed to regenerate on selective plates.

Colonies were allowed to grow for 7 days or until sporulation occurred. Two classes of transformant were recognised. Some sporulated rapidly (Class I). These were transferred both to fresh selective- and non-selective-V8 plates. A second class of transformant colony grew through the hygromycin B overlay to the surface and developed primordial pycnidia. However, these failed to mature and produced spores.

To assess the mitotic stability of some of the class I transformants, spores were harvested from a plug cut from non-selective plates and plated on V8 agar containing hygromycin B at concentrations of 1 mg/ml, 500 μg/ml, 250 μg/ml, 100 μg/ml, 50 μg/ml.

Four of these transformants were able to grow at all hygromycin B concentrations, albeit more slowly at the highest concentration, and one failed to grow at all (Fig. 1).

Analysis of the transformant and wild-type DNAs

Genomic DNA was isolated from three of the transformants, chosen from a total of fourteen recovered. The DNA was digested with two enzymes; BamH1, which cuts once within the plasmid pAN7-1, and EcoRV which has no sites. Genomic digests of the three transformants' DNA and wild type DNA, including undigested DNA of the transformants were blotted to nitrocellulose filters and hybridised to the photobiotinylated plasmid (Fig. 2). DNA from each of the transformants showed hybridisation to the probe. DNA from each of the transformants, when digested with BamH1, had two restriction fragments which hybridised with pAN7-1.

Transformant 1 (XF1), (Lane 2) showed bands of 12 kb & 5 kb. The intensity of the smaller band is approximately four times that of the larger whereas XF2 (Lane 3) and XF3 (Lane 4) showed two bands of 11.3 kb &

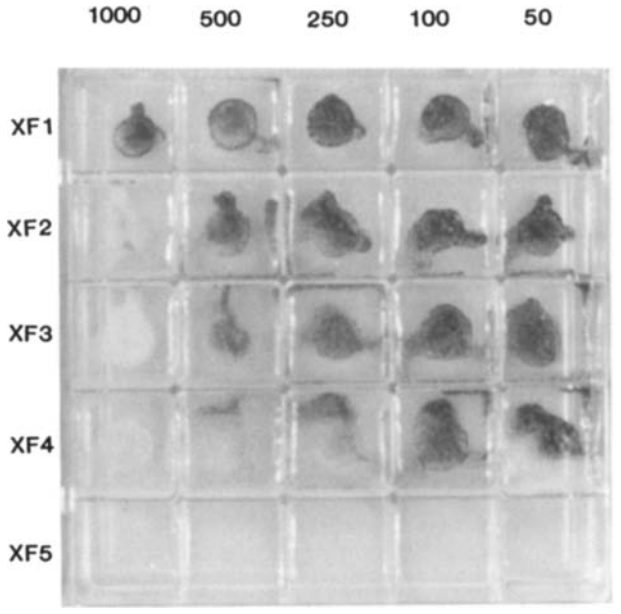

Fig. 1. Mitotic stability of transformants. Pycnidiospores were harvested from non-selective plates and plated on potato dextrose agar containing hygromycin B at concentrations of 1,000 μg/ml, 500 μg/ml, 250 μg/ml, 100 μg/ml and 50 μg/ml. Four of the first transformants to be analysed were able to grow on hygromycin B at concentrations of up to 1,000 μg/ml (top four rows). Transformant 5 shows mitotic instability. (Numbers indicate the concentration of hygromycin in μg/ml)

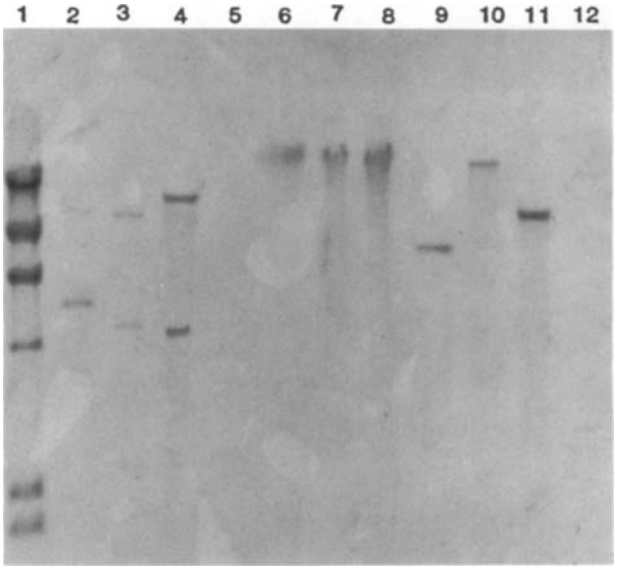

Fig. 2. Southern blot analysis of three transformants. Lane 1: photobiotinylated Lambda HindIII marker (migrates slightly slower than unlabelled Lambda cut with HindIII). Lanes 2,3 & 4: BamH1-digested transformants 1, 2 & 4, respectively. Lane 5: BamH1-digested wild-type. Lanes 6, 7 & 8: uncut DNA of transformants 1, 2 & 4, respectively. Lanes 9, 10 & 11: EcoRV-digested transformants 1, 2 & 4, respectively. Lane 12: EcoRV-digested wild-type DNA

4.5 kb; and 13.7 kb & 4.4 kb, respectively, with equal intensities.

Transformants DNA digested with EcoRV, each gave a single restriction fragment that hybridised to the probe. XF1 (Lane 9), XF2 (Lane 10) and XF3 (Lane 11) showed single bands of 9.2 kb, >30 kb and 11.5 kb, respectively. Undigested DNA from each transformant (Lanes 6, 7 & 8) showed hybridisation to the probe, corresponding to a size of >30 kb. Wild-type *Leptosphaeria* DNA digested with BamH1 (Lane 5) and EcoRV (Lane 12), showed no hybridisation to the probe. All restriction fragment sizes are approximate.

Discussion

Conditions for efficient protoplast release from *Leptosphaeria maculans* have been ascertained which allow rapid isolation of up to 10^8 protoplasts. Preliminary experiments indicated that regeneration of cell walls is rapid and rapid purification of the protoplasts is necessary for efficient transformation. The capacity for transformation decreases during the period that the protoplasts are held at room temperature prior to exposure to the DNA (data not shown).

Regeneration frequencies fluctuated, although frequencies between 5 and 10% have been repeatedly obtained. Of these, approximately 50% survived treatment with polyethylene glycol. After DNA treatment 3% of these regenerated on selective plates. The transformation frequency was, therefore, 3/2,000 protoplasts (60/μg DNA). However, only 14 out of approximately 3,000 transformants sporulated readily (defined as class I), and although the remainder of the "abortive" transformants (class II) could be encouraged to sporulate by subculturing repeatedly onto fresh selective plates, they were not bulked up. Preliminary experiments indicated that the time given for expression of hygromycin phosphotransferase before applying the selection may be critical for the establishment of sporulation in the transformants (i.e. producing class I transformants), since in one experiment which allowed 18 h before overlaying with hygromycin, all the transformants were of class II.

Southern blot analysis of three of the transformants revealed that a single copy of plasmid pAN7-1 had become integrated at a single site in the genome. In each of the transformants the integration was at a different site, indicating that random integration had probably occurred. The difference in intensity of the bands in the first transformant can be explained if the integration occurred close to the BamH1 site on the plasmid. This would give only a small region of homology on one fragment to which the probe could hybridise.

Confirmation of single copy can be achieved by sexual crossing; however the sexual cycle is slow (Musa

1981). The ease of protoplast production and handling suggests that protoplast fusion techniques may be applied to overcome this time problem.

Plasmid pAN7-1 transforms *Aspergillus nidulans, A. niger* (Punt et al. 1987), *F. fulva* (Oliver et al. 1987) and as we report here, *Leptosphaeria maculans.* This suggests that the *Aspergillus nidulans* GPDH promoter used in this vector will probably function in a large number of filamentous fungi. Turgeon et al. (1986), reported that a plasmid using a random *Cochliobolus heterostrophus* promoter has been used to transform *Leptosphaeria maculans.* Together, this implies that filamentous fungi are rather catholic in their acceptance of promoter sequences.

The ability to transform *Leptosphaeria* allows the molecular biology of its pathogenic interactions to be studied. Random integration of a plasmid into the genome will interrupt gene function. Genes involved directly in the pathogenicity of *Leptosphaeria* may be identified by the apparent loss of pathogenicity in transformants.

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